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GRANT NUMBER DAMD17-98-1-8230

TITLE: Role of the Wnt-4 Protein in the Mouse Mammary Gland

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REPORT DATE: July 1999

TYPE OF REPORT: Annual

PREPARED FOR: Commanding General
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1999	Annual (1 Jul 98 - 30 Jun 99)

4. TITLE AND SUBTITLE
Role of the Wnt-4 Protein in the Mouse Mammary Gland

5. FUNDING NUMBERS
DAMD17-98-1-8230

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The subject of the research described within this report is the mouse BRCA1 protein. The primary purpose of the experiments outlined below is to further the understanding of the role the mouse BRCA1 protein in cell proliferation and DNA damage. The scope of this research encompasses experiments that are designed to determine if the mouse and human BRCA1 proteins govern similar cellular functions and aims to identify novel regulatory components implicated in BRCA1-derived cancers through the use of a genetic model system in which mouse BRCA1 is mutated. Lastly, an immortalized mouse embryonic fibroblast cell line that harbors an exon 11 mutant form of Brca1 has been generated. This is significant because it provides a novel system which is suited for biochemical analysis of mouse Brca1 function.

14. SUBJECT TERMS
Breast Cancer, Brca1, cell cycle, DNA damage, genetic screen, genetic rescue

15. NUMBER OF PAGES
13

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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August 2.1999

PI - Signature Date

(4) Table of Contents

(5) Introduction

The subject of the research described within this report is the mouse BRCA1 protein. The primary purpose of the experiments outlined below is to further the understanding of the role the mouse BRCA1 protein in cell proliferation and DNA damage. Current evidence indicates that the human BRCA1 protein functions in biochemical pathways that are relevant to these processes. The emphasis of the research that involves the human version of the BRCA1 gene product conducted elsewhere is limited by a lack of a genetic system which can be manipulated with facility. The scope of this research encompasses experiments that are designed to determine if the mouse and human BRCA1 proteins govern similar cellular functions and aims to identify novel regulatory components implicated in BRCA1-derived cancers through the use of a genetic model system in which mouse BRCA1 is mutated.

(6) BODY

Specific Aims

1. Generation of a transgenic mouse that expresses human BRCA1.

Standard knockout strategies of mouse BRCA1 have resulted in lethality of embryos at a stage that precludes the opportunity to obtain cellular material for examination (1, 2). Approaches to study human BRCA1 frequently use methods which overexpress mutant versions of the protein in a wild type background. The inability to introduce mouse or human BRCA1 or mutant versions of their gene products into a genetic background harboring null alleles of BRCA1 significantly hinders the ability to study these proteins in what is arguably a more biologically relevant context. A strategy we have selected is to try to rescue the embryonic lethality of mouse BRCA1 knockout embryos with a transgenic model in which human BRCA1 expression is conferred by the mouse cyclin E promoter. The reasoning for this is as follows. The mouse cyclin E promoter has been demonstrated to control reporter gene expression in a cell cycle dependent manner (3). Published results indicate that human BRCA1 is regulated accordingly, and data from our laboratory indicates that this feature is conserved by the mouse protein (4-6, unpublished). The ubiquitous expression of mouse BRCA1 during early embryogenesis is similar to mouse cyclin E expression and cyclin E mRNA expression is substantially decreased in BRCA1 knockout embryos suggesting that there is overlap in the cell types that are dependent upon expression of BRCA1 and cyclin E (1, 7). The construct to achieve the desired temporal and spatial expression of human BRCA1 is shown in Figure 1.

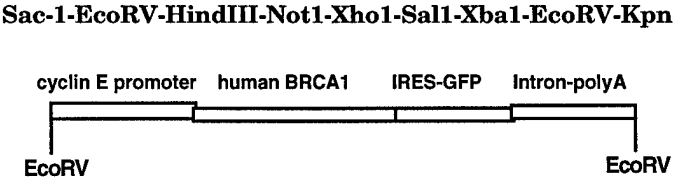

Figure 1. A polylinker containing the sites Sac1-EcoRV- HindIII-Not1-Xho1-Sal1-Xba1-EcoRV-Kpn1 was constructed to replace the existing Bluescript KS polylinker. The order of subcloning of the fragments is as follows. A 1.8 kb fragment containing an intron and polyadenylation signal known to be effective in conferring expression on several transgene we inserted into the Xba1 site. The human cDNA for BRCA1 is cloned into the HindIII-Not1

sites. A 1 kb promoter region of mouse cyclin E is blunted and cloned into the single blunted HindIII site. Lastly, a 1.4 kb Xho1-Sal1 fragment containing IRES-GFP sequences was inserted into the corresponding sites in the vector. The 11.5 microinjection fragment, referred to as CEB, was excised with EcoRV.

Microinjection of the CEB construct yielded 13 positive founder lines identified by PCR analysis of genomic DNA derived from tail samples. Results were confirmed with independent primer sets encompassing regions within the human BRCA1 cDNA and the 3' intron-polyadenylation sequences. Expansion of the founder lines is currently underway. Embryos will be obtained from crosses of wild-type and transgenic animals and embryos will be screened by RNase protection analysis to identify lines in which the expression of human BRCA1 is detected. These lines will be inter-crossed with the BRCA1 mouse knockout line to generate bitransgenic animals (mice heterozygous for BRCA1 that harbor the CEB transgene) which will subsequently be crossed to BRCA1 heterozygous animals. The ability of human BRCA1 to rescue the knockout phenotype will be determined by dissecting litters derived from bitransgenic and BRCA1 heterozygous crosses at approximately day 9.5 of development which will be identified by genotyping. In the mouse knockout strain used for these experiments a BRCA1 knockout has never been found to be viable past embryonic day 5.5, therefore the presence of the transgene in an embryo that is also found to be BRCA1 null would be indicative of a rescued phenotype. The predicted endpoints are as follows. In a complete rescue cyclin E-directed human BRCA1 expression is coincident with all sites of mouse BRCA1 expression and the human protein albeit genetically disimilar is able to phenocopy the functionality of the mouse version. The conditions of rescue can be defined by the ability of the transgene to rescue developmental processes and extended to the requirement of BRCA1 in adult tissues. Alternatively, cyclin E promoter expression is not directed to a subset of cells or tissues that have an absolute requirement for BRCA1 expression for continued development. It is possible that mice will survive a few days longer as compared to the BRCA1 null. This scenario has the potential to provide the opportunity to study BRCA1-/- cells as well as rescued cells especially in an *in vitro* context. Lastly, transgene expression levels may be too low to activate the appropriate signaling pathways, the human protein may be functionally divergent from the mouse

version, or transgene transcript is not translated efficiently. Given the first two possibilities it will be interesting to conduct experiments that examine more closely the ability of the human protein to complement the function of mouse BRCA1. Specific questions will focus on determining whether all or only a subset of the processes associated with BRCA1 function can be complemented. Experiments will be conducted to determine if proliferation, DNA damage, and differentiation occur in a manner that is indistinguishable from wild-type.

2. Specific Aim 2: Identification of genes regulated by mouse Brca1 expression.

High-density oligonucleotide arrays have been used successfully to identify target genes whose expression may be induced or repressed in the presence of a protein of interest. Using a Cre-loxP system mice have been generated in which a specific germline deletion of exon 11 of murine BRCA1 has been created (8). We have undertaken a study in which arrays containing 6500 known murine transcripts and expressed sequence tags will be hybridized to mRNA generated from mouse embryo fibroblasts (MEFS) derived from wild-type and exon 11 mutant BRCA1 mice. Experiments have determined that MEFS from these mice are defective in a G2-M cell cycle checkpoint in response to γ-irradiation. This data suggests that there are candidate genes that may be either repressed or induced in the presence of Brca1 that play a role in cellular responses including sensing DNA damage, activating components that mediate cell cycle arrest, or direct involvement in DNA repair. Brca1 has been implicated in transcriptional activation and repression and use of the gene chip technology has identified genes that respond to an increase in Brca1 expression (9). At the present time a screen for genes that are differentially expressed in response to DNA damage in the context of a genetic background that lacks a fully functional BRCA1 protein has not been conducted. It is likely that elements of pathways that are governed by Brca1 function will be identified. MEFS from wild-type and exon 11 knockout embryos will be treated with 3 grays of radiation and samples will be harvested 1 hour post-irradiation for processing for array hybridization. Candidate genes that are differentially expressed in wild type as compared to knockout MEFS in response to treatment with radiation will be analyzed by Northern analysis to corroborate results obtained with the chip technology.

3. Specific Aim 3: DNA damage and cell cycle checkpoint in exon 11 Brca1 knockout mouse embryo fibroblasts.

Mouse embryo fibroblasts derived from Brca1 exon 11 deletion mutants undergo senescence during the first few passages in culture (8). A plate of these fibroblasts maintained in culture and refed every 2-3 days for a period of three months fortuitously gave rise to apparently immortalized cell lines. Brca1 exon 11 mutant cells have a defect in the G2-M checkpoint following treatment with γ-irradiation and MMS but not to UV. This suggests that the region encoded by exon 11 may be involved directly with the process of DNA repair of specific lesions or as a component of the cell cycle machinery to impose cell cycle arrest in response to the detection of these lesions. Experiments using cells in which human Brca1 protein is truncated have shown that an intact BRCT domain is necessary for transcription-coupled repair, a repair pathway that is also affected in mouse embryonic stem cells deficient in Brca1 (10). Importantly, rad51, a protein associated with double-stranded break repair and homologous recombination binds to the region of Brca1 that is encoded by exon 11 (11).

In order to define the nature of the lesions that contribute to the exon 11 knockout phenotype cells will be subjected to DNA repair assays. Homologous recombination, considered to represent a measure of double-stranded break repair, can be be measured by transfection of distinct plasmids that contain either the 5' or the 3' region of a gene that when recombined yield an expression plasmid that confers resistance under selection or a measurable activity such as chloramphenicol-acetyl transferase (12, 13). Although the specific mechanisms governing the joining process that leads to the formation of the recombined molecules are unknown, there are two models, one known as the double-stranded break repair and the second referred to as the single-stranded annealing model (13). At this time a role for the Brca1 protein in pathways governed by either of these models has not been ruled out. Substrates that are predicted to form a single molecule upon homologous recombination will be designed as follows. The plasmid pGL3 Control contains SV40 promoter sequences that direct expression of the firefly luciferase gene and serves as the DNA template from which the substrates will be derived. This plasmid will be modified to create two distinct plasmids that separately contain the 5' end and the 3'end of the luciferase gene respectively. Linearized versions of these plasmids and control plasmids will be transfected into wild-type and knockout cells and cell lysates will be

harvested 48 hours post-transfection and assayed for firefly luciferase activity. Transfection efficiency will be normalized by cotransfection of the plasmid pRL-CMV that contains the SV40 promoter directing expression of renilla luciferase.

Brca1 embryonic stem cells are defective in transcription-coupled repair of thymine glycol lesions that are the result of oxidative damage to DNA (10). The recent availability of thymine glycol antibodies that recognize and specifically immunoprecipitate thymine glycol-DNA lesions provides the opportunity to determine if Brca1 exon 11 is required for repair of these lesions. MEFS will be treated with a range of doses of UV, hydrogen peroxide, and γ-radiation and harvested to obtain genomic DNA at 30 minutes and 1 hour following exposure to DNA damaging agents. DNA will be digested with BamH1 and incubated with thymine glycol antibody. Samples will be subjected to Southern blot hybridization with strand specific probes of sequences derived from the mouse dihydrofolate reductase gene.

The experiments described up to this point have not addressed whether or not the cell cycle checkpoint defect and other features of the knockout phenotype are reversible through gene transfer of wild-type Brca1 into mutant cells. This is an important question for two reasons. First, if Brca1 is able to return the knockout cells to a state that approximates that of wild-type cells, it suggests, but does not prove, that the observed defects occur as a direct result of the loss of Brca1 function as opposed to events that occurred in a stochastic manner, over time such as gain of function or loss of funtion mutations in other genes. Secondly, if specific attributes of the phenotype are reversible it will be possible to introduce mutant versions of Brca1 that contain deletions or point mutations in defined regions within the exon 11 region to more precisely map critical domains. To this end, mouse Brca1 will be cloned into the MIGR1 retroviral vector, transiently transfected into the BOSC producer cell line and supernatants harvested for infection of mouse Brca1 exon 11 mutant fibroblasts. Cells will be analyzed to determine if the cell cycle checkpoint and any DNA repair defects associated with the loss of Brca1 are corrected upon introduction of wild-type protein.

Statement of Work

Task 1: Generation of a transgenic mouse model that expresses human BRCA1: months 1-36

- Construction of transgenic construct for microinjection: months 1-6
- Breeding of transgenic lines for harvest of genomic DNA and screening for founder lines: months 6-12
- Breed transgenic mice into Brca1 heterozygous background: months 12-18
- Breed bitransgenic animals with Brca1 heterozygous mice: months 18-24
- Analyze complementation capacity of human BRCA1 in mouse cells by cell cycle analysis and DNA repair assays: months 24-36

Task 2: Identification of genes regulated by mouse Brca1 expression: months 1-36

- Generation of mRNA from mouse embryo fibroblasts: months 1-6
- Preparation of mRNA for hybridization and application to test chips: months 6-12
- Hybridization of samples to arrays and analysis of results: months 12-18
- Analysis of candidate genes by Northern blot analysis: months 18-24
- Experiments designed to demonstrate functional relevance of candidate genes with respect to Brca1 function: months 24-36.

Task 3: DNA damage and cell cycle checkpoint in exon 11 Brca1 knockout mouse embryo fibroblasts: months 12-36

- Construction of plasmids for homologous recombination assay: months 12-16
- Transfection of cell lines to assay for homologous recombination: months 16-24
- Transcription-coupled repair of thymine glycol lesions in MEFS: months 16-24
- Generation of retroviral constructs for infection of MEFS: months 18-24.
- Experiments to determine if Brca1 mutant phenotype is reversible: months 24-36.

(7) Key Research Accomplishments

- Generation of transgenic mouse lines that express human BRCA1.
- Generation of immortalized exon 11 mutant Brca1 mouse embryo fibroblasts for analysis of cell cycle and DNA damage.

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